(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 November 2003 (06.11.2003)

PCT

(10) International Publication Number
WO 03/090546 A1

(51) International Patent Classification[7]: A23C 9/127, 19/032 // (C12N 1/20, C12R 1:225, 1:23)

(21) International Application Number: PCT/SE03/00632

(22) International Filing Date: 17 April 2003 (17.04.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0201214-4 23 April 2002 (23.04.2002) SE

(71) Applicant and
(72) Inventor: **MAHDAVI, Jafar** [SE/SE]; Brotorpsgatan 10C, S-507 65 Borås (SE).

(74) Agent: **ALBIHNS STOCKHOLM AB**; Linnégatan 2, P:O. Box 5581, S-11485 Stockholm (SE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/090546 A1

(54) Title: MULTICULTURAL FERMENTED YOGURT

(57) Abstract: The invention relates to a fermented milk product such as yogurt, butter or cheese comprising bacteria from the Lactobacillus species and yeast and having a galactose content of less than 1,2 g/l, a lactose content of less than 1,2 g/l, and lactic acid content of at least 0.2 weight %. It also relates to a two step method for producing fermented yogurt with Lactobacillus bacteria and yeast. Further, it concerns a pharmaceutical or probiotic composition comprising the fermented milk product, use of such a fermented milk product for the production of a pharmaceutical for lowering of plasma triglycerides and lipids, for hampering cell death and fatty-liver, especially caused by the consumption of alcohol, for profylax and treatment of atherosclerosis and coronary heart disease and for profylax and treatment of lactase and galactase deficient individuals. It also concerns a symbiotic or functional food product comprising the fermented milk product.

MULTICULTURAL FERMENTED YOGURT

The present invention relates to a fermented milk product such as yogurt, butter or cheese comprising bacteria from the Lactobacillus species and yeast and a lactose content of less than 10 g/l and a lactic acid content of at least 0,2 weight %. It also relates to a unique two-step method for producing fermented yogurt with Lactobacillus bacteria and yeast. Further, it concerns a pharmaceutical or probiotic composition comprising the fermented milk product, use of such a fermented milk product for the production of a pharmaceutical for lowering of plasma triglycerides and lipids, for hampering cell death and fatty-liver caused by the consumption of alcohol, for profylax and treatment of atherosclerosis and coronary heart disease and for profylax and treatment of lactase and galactase deficient individuals. It also concerns a symbiotic or functional food product comprising the fermented milk product

Technical background

Alcohol, at least in industrialised countries is perhaps the most common cause of liver cirrhosis. Alcohol is a hepatotoxin that affects mitochondrial and microsomal functions, where part of the hepatotoxic effects could be attributed to enzymatic oxidation of ethanol to acetaldehyde. The ethanol metabolism has furthermore been implicated in increased mobilisation of free fatty acids, diminished utilisation of triglycerides, decreased oxidation of fatty acids, increased esterification and reduced lipoprotein excretion (Niemela et al., 1998). Free radicals generated from oxidative stress can remove electrons from unsaturated fatty acids resulting in lipid radicals. The lipid radicals can react with oxygen to form lipid peroxide radicals which, in turn, interact with other fatty acids, thereby creating a chain reaction of lipid peroxidation. Such chain reactions may generate biologically active compounds, such as molecules that cause widening or narrowing of blood vessels, thereby increasing the risk of cardiovascular disease (Ponnappa et al., 2000).

In general *Lactobacillus* cultures possess low lipase activity. The lipids in fermented yogurt product are partially degraded and thus the digestibility of hydrolysed fermented

yogurt fat is higher. According to Blanc et al., 1973, there is a 2.5-fold increase in free acids in natural yogurt compared to milk. Rasic et al., 1978, concluded that the lipase activity processed by lactic acid bacteria in yogurt favourably influences the dietetic value of the final fermented product.

Intracellular accumulation of cholesterol and cholesterol esters are prominent also in non-alcohol related diseases, where atherosclerosis would be the most important disease. Results from several studies indicate that the consumption of yogurt reduces the serum cholesterol levels. Mann et al, 1972, found that serum cholesterol levels in African men decreased after consumption of yogurt. In another study by Mann et al, 1977, yogurt was found to have a hypocholesterolemic effect on Caucasians, where incorporation of C_{14}-acetate into cholesterol was reduced during yogurt ingestion, suggesting that the yogurt products inhibit cholesterol synthesis.

It has now turned out that a milk product that has been fermented in a first step with lactobacilli and in a second step, preferably after dilution(s) with lactobacilli and yeast comprises a low content of lactose and a high content of lactic acid compared to ordinary yogurt. Yogurt and butter and cheese produced thereof have beneficial effects on pharmacokinetics of alcohol. Thus, these products protect epithelial cells against exposure to alcohol, reduce the blood alcohol concentration (BAC) and lower the plasma lipid content.

USP 4 034 115 and EP 0 122 104 disclose a two and three step respectively fermentation process of milk with lactobacilli in order to break down lactose in milk. No yeast is used and there is no information about any effect on pharmacokinetics of alcohol. Tarhana is a fermented product comprising yogurt and yeast but also cereal and vegetables such as tomatoes, onions, green pepper etc., Department of Food Engineering, Trakya University, Agriculture Faculty of Tekirdag, Turkey, 2000.

Summary of the invention

The present invention relates to a fermented milk product such as yogurt, butter or cheese comprising bacteria from the Lactobacillus species and yeast and having a galactose content of less than 1,2 g/l, a lactose content of less than 1,2 g/l, and lactic acid content of at least 0.2 weight %. It also relates to a two step method for producing fermented yogurt with Lactobacillus bacteria and *yeast*. Further, it concerns a pharmaceutical or probiotic composition comprising a fermented milk product, comprising bacteria from the Lactobacillus species and yeast, use of a fermented milk product for the production of a pharmaceutical for lowering of plasma triglycerides and lipids, for hampering cell death and fatty-liver caused by the consumption of alcohol, for profylax and treatment of atherosclerosis and coronary heart disease and for profylax and treatment of lactase and galactase deficient individuals. It also concerns a symbiotic or functional food product comprising the fermented milk product

Due to the additional fermentation step in the final fermented yogurt product, the level of lactic acid is higher and the lactose content is significantly lower, compared to conventional yogurt (Example 6 and 7).

The influence by lactic acid from the yogurt according to the invention is demonstrated (see below). In addition, bacterial alcohol-dehydrogenase (ADH) and acetaldehyd-dehydrogenase (ALDH) are produced, which decrease the bioavailability of ethanol by production of acetaldehyde andacetate.

Addition of fermented yogurt suspension or lactic acid to human epithelial cells (KB) diminished ethanol-induced cell-death (Example 2). The fermented yogurt end product reduces ethanol (Example 4) and triglycerides levels in serum.
The fermented yogurt products cause delayed uptake of ethanol in the gastrointestinal (GI) tract and a reduction in systemic ethanol levels. The regular intake of the fermented products may attenuate development of fatty-liver caused by extensive consumption of

ethanol and in addition, stabilise Atherogenic Index (AI, explained in page 20), which would be beneficial for prevention of atherosclerosis and coronary heart disease.

Thus, the products according to the present invention are novel preventive and therapeutic agents for combating disorders caused by excessive alcohol intake, and/or elevated blood-lipids levels. They can be used for the reduction of total plasma cholesterol and serum ethanol concentration thus lowering the incidence of coronary heart disease and fatty liver disease, respectively.

Detailed description of the invention

The present invention relates to a fermented milk product comprising bacteria from the Lactobacillus species and yeast and a galactose content of less than 1,2 g/l, a lactose content of less than 1,2 g/l, and lactic acid content of at least 0.2 weight %.

The expression "comprising" refers to the fact that also other ingredients may be present in the product.

Preferably the galactose content is less than 1,2 g/l (0,12%) and the lactose content less than 0,4 (0,04%) g/l and most preferred the galactose content is less than 0,4 g/l (0,04%) and the lactose content of less than 0,4 g/l (0,04%).

The pH of the milk product is below 4,5, especially below 4,1 and most preferred below 3,5 and especially 3,4.

The yeast may be any yeast such as Kluyveromyces thermotolerans, Pichia fermentans and Saccharomyces cerevisiae. Preferably the yeast is chosen from *Kluyveromyces marxianus*/Hansen Van der Walt, *kafir 14,* DSM 14502.

The Lactobacillus species may be chosen from the genus *Lactobacillus* and *Streptococcus*, preferably *L. helveticus* (AD4550/01 DSM 14492) and *L. acidophilus* (Lb. 67 DSM 14499).

The fermented milk product according to the invention may be any dairy product such as yogurt, butter, cottage cheese or cheese.

The invention also relates to a method for producing a fermented yogurt, characterised in that milk is heated, Lactobacillus bacteria are added and the bacterial suspension is incubated at 25-30, preferably at 37°C for 2-24, preferably 12 hours in normal atmosphere, the yogurt produced is preferably diluted with water and additional Lactobacillus bacteria and in addition, yeast is added and the suspension is incubated at 15-25 °C, preferably at room temperature for 1- 15, days, preferably 7 days in a closed system.

Fermentation is here defined as processes that release energy from carbohydrates but do not require oxygen.

According to the invention a multi-cultural fermented yogurt is made using at least one preferably at least two *Lactobacillus* strains in combination with at least one yeast strain by processes that utilise the disaccharide carbohydrate lactose, which is naturally present in yogurt. The *Lactobacillus* strains and yeast strain respectively may be the same or different strains.

The milk, that is used as starting product, may be any type of milk, such as natural and untreated milk, pasteurised and homogenised milk of low fat type, standard type or with the natural content of fat.

The milk is warmed to 25-120°C, preferably 35-110 °C, especially 85-105°C and most preferred to 100°C for at least 5 minutes, preferably at least 10 minutes and especially for about 15 minutes.

A culture of bacteria is added. The bacterial suspension is then incubated at 25-50 °C, preferably 37°C for at least 5 hours, preferably 7-20 hours and most preferred 12 hours in normal atmosphere.

The yogurt then produced is diluted such as with water. It has turned out that the second fermentation process is dependent on dilution. 1 volume yogurt may be added to 0,2-10 volumes of water, preferably to 0,5-6 volumes of water, especially to 1-4 volumes of water and most preferably to 2 volumes of water, i.e. a dilution of 1/1.2-1/11 vol./vol., preferably 1/1,5-1/7 vol./vol., especially 1/2-1/5 vol./vol. and most preferred 1/3vol./vol. Additional Lactobacilli bacterial cells diluted 1:1000 and yeast diluted 1:10000 of cultivated cell cultures are added. The suspension is then incubated at room temperature (20-30°C) for at least 24 hours, preferably 2-15 days, preferably one week in a closed system.

The dilution with water, that is a dilution step without the addition of nurturance (such as milk-product) will drive the fermentative processes further, which promotes the formation of beneficially substances and effects as described below, where the unique combination of low lactose and galactose continuance qualitative characteristic of the products.
The end product comprises about 15%, especially 5-10% living lactobacillus strains. It is believed that this is due to the degradation of both lactose and galactose.

The product may be kept in room temperature for more than a year and still keep its qualities.

Any additives such as stabilising, thickening and/or flavouring agent may be added. To compensate for the taste of fermented yogurt preferably sodium chloride (e.g. 0.08% w/v NaCl) is added. As thickener hydroxymethyl cellulose may be used.
As stabilisers gelatine, vegetable gums, carboxymethyl cellulose, locust bean, guar and seaweed gums like alginates and carrageenans.

The multi-cultured yogurt is then further fermented for about one week. Then the churning cream in form of butter may be separated from the fermented suspension by shaking at 30° for 24 hours. The butter consists of 95% fat and live bacteria and yeast.

The invention relates to any type of cheese such as cottage cheese made from the multicultural milk product of the invention according to well established methods in industry.

The content of lactose in the product is less than 10 g/l, preferably less than 5 g/l, especially less than 2 g/l such as less than 1g/l and especially less than 0,8 g/l. Normally the lactose content in yogurt and lactose free milk is between 10-30 g/l respectively 1-10 g/l (according to the Swedish national food administration). Most preferably the lactose content is less than 1 g/l and preferably less than 0.05 g/l, especially less than 0.4 g/l and most preferably less than 0.4 g/l.

The lactic acid content of the multi-cultural product is at least 0.2 weight % preferably at least 0.3 weight %, such as at least 0.4 weight %. The content is typically around 0.3-0.6, especially 0.4-0.5 weight %. This is more than in the casual yogurt, which contains D(-) lactic acid (0.05 g/100g) and L(+) lactic acid (0.07 g/100g) in total 0.12 weight %, compared to the fermented yogurt which contain higher amount especially of the D (-) isomer, around 0.5 g/100g).

The invention also relates to a pharmaceutical or probiotic composition comprising a fermented milk product, comprising bacteria from the Lactobacillus species and yeast having a lactose content of less than 1,2 g/l and especially less than 0,8 g/l and a lactic acid content of at least 0.2 weight %.

The invention also relates to a pharmaceutical or probiotic composition comprising a fermented milk product, comprising bacteria from the Lactobacillus species and yeast having a galactose content of less than 1,2 g/l, especially less than 0.4 g/l.

One aspect of the invention is the use of a fermented milk product according to the invention for the production of a pharmaceutical for lowering of plasma triglycerides and lipids.

Another aspect of the invention is the use of a fermented milk product according the invention for the production of a pharmaceutical for hampering cell death, especially caused by the consumption of alcohol.

A further aspect of the invention is the use of a fermented milk product according to the invention for the production of a pharmaceutical for hampering fatty-liver, especially caused by the consumption of alcohol.

Still another aspect of the invention is the use of a fermented milk product according the invention for the production of a pharmaceutical for profylax and treatment of atherosclerosis and coronary heart disease.

The invention also covers the use of a fermented milk product according the invention for the production of a pharmaceutical for profylax and treatment of lactase and galactase deficient individuals.

The invention also concerns a symbiotic or functional food product comprising the fermented milk product

The invention will now be described with the help of the following figures:

Figure 1A. Analysis of the effect of ethanol and fermented yogurt in a dilution series on human epithelial cells. Spots staples indicate the effect of ethanol on epithelial cells and sphere staples indicate the protective effect of fermented yogurt-supernatant (v/v). The error bars indicate the percentage error amount (± 5%). FY; Fermented Yogurt.

Figure 1B. Analysis of the protective effect of lactic acid mixed with a dilution series of alcohol on human epithelial cells. Spots staples indicate the protective effect of ethanol on epithelial cells and outlined diamond indicate the effect of 0.4 lactic acid (v/v) in the presence of ethanol with increasing dilution. The error bars indicate the percentage error amount (± 5%). LA; Lactic Acid.

Figure 1C. Analysis of the protective effects of fermented yogurt mixed with a dilution series of alcohol on human epithelial. Spots staples indicate the effect of ethanol on epithelial cells and horizontal brick indicate the protective effect of fermented yogurt-supernatant (v/v) in the presence of ethanol with increasing dilution. The error bars indicate the percentage error amount (± 5%). FY; Fermented Yogurt.

Figure 2A. The protective effect of the fermented yogurt on the blood alcohol concentration (BAC), in one healthy man after oral administration of 62.5 ml ethanol (40% v/v) diluted with 62.5 ml orange juice. 45 minutes after intake of alcohol, the blood samples was obtained. The Figure shows the corresponding concentrations of serum ethanol after drinking of different quantities of fermented yogurt. Dashed vertical staples indicate the negative control (300 ml milk) and shingle staples show different intake of fermented yogurt in ml. The error bars indicate the percentage error amount (± 2.5%). Al; Alcohol, FY; Fermented yogurt.

Figure 2B. Serum alcohol profile in three healthy subjects (I, II and III), who consumed 62.5 ml ethanol (40% v/v) diluted with 62.5 ml orange juice, together with 300 ml fermented yogurt in exactly 30 minutes after standard breakfast. 45 minutes after drinking end, samples of blood were obtained. The spot staples indicate serum alcohol profile in combination with milk (300 ml, as control), and shingle staples are corresponded alcohol profile in combination with fermented yogurt. The error bars indicate the percentage error amount (± 2.5%). FY; Fermented Yogurt.

Figure 3. Serum alcohol profile in two healthy subjects (I and II), who drank 250 ml ethanol (40% v/v) in two different occasions in exactly 3 hours immediately after dinner.

In the first occasion 500 ml milk and in the second occasion 500 ml fermented yogurt (shingle staples), after end of drinking. After eight hours after drinking end, samples of blood were obtained. The error bars indicate the percentage error amount (± 2.5%). Al; Alcohol, FY; Fermented Yogurt.

Figure 4. Serum-triglyceride concentration profile for five healthy volunteers (I, II, III, IV and V) before and after treatment with fermented yogurt/butter. One person, who had normal diet during experiment (I), was used as control, and two persons (II and III) consumed 45 g fermented butter diurnal. IV and V represent the persons who consumed one litre-fermented yogurt. Spot staples indicate triglyceride concentration before and shingle staples after treatment. R; Reduction, Ine; Increase.

Figure 5. Atherogenic Index (AI) profiles for 5 healthy persons (I, II, III, IV and V), who consumed either one litre fermented yogurt (II and III) or 45 gram fermented butter diurnal (IV and V). One person (I) with normal diet during the experimental period (30 days) was used as control. Spot staples indicate AI before and shingle staples after treatment. FY; Fermented Yogurt, FB; Fermented Butter.

The invention will now be described more in detail referring to the following non-limiting examples.

Example 1. Fermentation of yogurt

Lactobacillus and growth conditions

For production and fermentation of yogurt a bacterium of the Lactobacillus species, preferably *Lactobacillus acidophilus* (Lb 67 DSM 14499), *Lactobacillus helveticus* (AD4550/01 DSM 14492) and *yeast (Kluyveromyces marxianus/*Hansen Van der Walt, *kafir 14,* DSM 14502*)*, were grown in MRS broth (Difco) or Rogosa plates, in 37°C with gentle shaking for 24 hours.

Fermentation process

To one litre of standard milk (3% fat) boiled to 100°C for 15 minutes, $4*10^9$ "colony forming units" (CFU) of cultivated bacterial (*L. helveticus*, grown in MRS broth) was added. The milk suspension was then incubated in 37°C for 12 hours in normal atmosphere. The yogurt then produced was diluted with water (1:3 or 1 L yogurt and 2 L water) and additional bacteria and yeast cells (*L.acidophilus* $4*10^9$, and *Kluyveromyces marxianus*, $1*10^9$, per one litre yogurt suspension) of cultivated cell cultures was added. The suspension was then incubated at room temperature for one week in a closed system. To compensate for the taste of fermented yogurt Sodium Chloride (0.08% w/v NaCl) was added.

Example 2. Fermented yogurt protects epithelial cells against exposure to alcohol

Cytotoxicity assays

To determine the cytotoxic effect of ethanol and protective effects by fermented yogurt or lactic acid on KB cells (final concentration in the assay mixture 4-8%), several assays were used. The combinations of 8%-ethanol (v/v) and 8%-fermented yogurt suspension (v/v) or 0.4% lactic acid (v/v) were incubated at 37°C for 24 h before use. The pH of lactic acid solution was adjusted to 4.9 before incubation with ethanol. Changes in cell morphology and detachment from the underlying surface were monitored by light microscopy at 400 X magnification. For estimation of the uninjured cells attached to the surface of the culture well, the neutral red uptake assay was used. For this purpose, fresh confluent cell layers in a 96-well microtiterplate were incubated at 37°C for up to 4 h in culture medium (E-MEM with antibiotics and glutamine or keratinocyte growth medium) with 5 mM L-cysteine. The medium was replaced by 0.1 ml D-MEM-medium, containing 10% fetal calf serum. Then, medium was incubated at 37°C with 40 µg/ml neutral red for 2 h. The uptake of neutral red by the cells was measured at 540 nm after fixation and solubilisation of the cells in 50% ethanol with 1% acetic acid.

The epithelial cell cultures

The target cells used were human epithelial cell culture (KB-line, CCL 17, Flow Laboratories, Glasgow, UK) grown at 37°C in the presence of 5% CO_2.

KB cells were grown in Eagle minimum essential medium (E-MEM) supplemented with 20,000 U/l penicillin, 100 µg/l streptomycin, 0.2 mg/l L-glutamine and 10% fetal calf serum were added. Then, cell culture medium was enriched with non-essential amino acids (10 ml/l from a stock solution). The growth medium was changed every 48 h. The KB cell cultures in the cytotoxicity experiments were in their third to eighth passage used as the target cells.

Chemicals

E-MEM, L-glutamine, and fetal calf serum were purchased from Flow Laboratories, Glasgow, UK. Keratinocyte growth medium was from Promocell, Heidelberg, Germany. Proteinase inhibitors and N-α-benzyl-DL-arginine *p*-nitroaniline (BA*p*NA) were obtained from Boeringer Mannheim, Germany. Bovine serum albumin, antibiotics, non-essential aminoacids (100x concentrated stock solution), metal salts, Azocoll and other compounds were obtained from Sigma Chemical Co., St. Louis, MO, USA.

The epithelial cell lineage KB, was established and used to investigate the possible cell protective effects of sterile filtrated fermented yogurt on alcohol exposure *in vitro*. Fermentation results in conversion of the lactose into lactic acid and thereby contributes to acidity (pH 4.9) of the product. Pre-digestive and enzymatic activities by *Lactobacillus* and *yeast* strains in combination with low pH could possibly have negative effects on cell-growth. In Figure 1A, the epithelial cells were exposed to either alcohol or fermented yogurt in dilution series and cell viability was analysed. There was no significantly reduction in viable cells due to exposure to fermented yogurt only.

Probably, fermented yogurt contains several bio-active compounds, with inhibitory effects on alcohol. The presence of lactic acid in fermented yogurt is desirable since it acts as a natural preservative and thereby contribute to the biological safety of the product, therefore, the bio-protective effects of lactic acid against alcohol was analysed. The results (Fig 1B) demonstrate that lactic acid in low concentration (0.4, pH 4.9) protects the epithelial cell viability during alcohol exposure.

Finally, the original suspension of fermented and edible yogurt was evaluated for protective effects facing alcohol exposure. In Fig 1 C, the epithelial cells were exposed to alcohol together with fermented yogurt product. Interestingly, the results show congruity as in Fig B, where toxicity of alcohol is negligible in presence of lactic acid.

Here, ethanol possibly due toesterification with lactic acid (Fig 1B) or constituents of fermented yogurt such as amino acids glycine and alanine became undetectable in the blood as free ethanol. The similar effects by amino acids has been described by Widmark, 1933, Widmark, 1936; Neymark and Widmark, 1941). Long-term fermentation of a multi-cultured yogurt cause low pH due to lactic acid and consequently, lysis of the bacterial cells. Thereby, high levels of endogenous ADH and ALDH enzymes are released in to the GI-tract and available for inactivation of ethanol.

Example 3. Blood alcohol concentration (BAC) in one individual after oral administration of ethanol together with different volumes of fermented yogurt

Determination of blood ethanol

Venous blood samples obtained from an indwelling catheter were used for determination of blood ethanol. The blood was drawn into 5 ml Vacutainer tubes containing Sodium fluoride (20 mg, NaF) and heparin (143 units). Aliquots of blood-serum (100 µl) were removed and diluted 11-fold with n-propanol (8 mg/l) as an internal standard. The serum and internal standard were injected into head space sampling vials (22 ml), which were immediately made air-tight with rubber stoppers and crimped-on aluminium caps. For gas chromatography, a glass column packed with Carbopack C (0.2% Carbowax 1500 on Carbopack 80-100 mesh) was used as the stationary phase. The analytical precision expressed as the standard deviation of a single determination increases with the concentration of ethanol in the samples. The limit of quantification for this method is approximately 0.22 mmol/l (1 mg/l), (Jones, 1991).

Figure 2A shows blood alcohol concentrations (BACs) from a series of experiments. Here, the volunteer consumed 62.5 ml of ethanol (40% v/v, diluted to 20% v/v with orange juice) supplemented with milk or fermented yogurt. In the control experiment, the volunteer drank an identical volume of ethanol but supplemented with 300 ml of regular milk, a mixture which did not confer any reduction in BAC compared to the positive control (Fig 2A, milk). In the next series of sessions, the same volunteer again consumed the identical volume of alcohol, but in combination with increasing volumes of fermented yogurt product. Blood samples were obtained 45 minutes after start of intake of ethanol. The results suggest that already 100 ml of fermented yogurt product significantly reduced BAC, while consumption of 300-400 ml of yogurt product can further reduce BAC.

It was found that consumption of ethanol with fermented yogurt, regardless of the nutritional composition of the meal, caused a pronounced lowering of the peak BAC compared with casual yogurt or milk in three subjects (Fig. 2B). It is believed that the reduction in BAC for the consumption of fermented yogurt compared to milk can be explained by two mechanisms. First, fermented yogurt contains high level of lactic acid, which can esterfy ethanol. Ethanol has here shown to undergo esterification with lactic acid (Fig 1B) or constituents of fermented yogurt such as amino acids glycine and alanine and therefore become undetectable in the blood as free ethanol. Second, long-term fermentation of a multi-cultured yogurt cause low pH by lactic acid and consequently, lysis of the bacterial cells. Thus, high levels of endogenous ADH and ALDH enzymes are released.

Example 4. The effect of fermented yogurt on BAC in three volunteers

Effect of fermented yogurt on alcohol pharmacokinetics

The protective effect of pre-treatment of fermented yogurt was analysed in three volunteers. Here the higher level of fermented yogurt was chosen, i.e. the 300 ml volume, and ingested with ethanol during a 45-minut test period.

Three healthy male and female students with a mean age 25.3 yr. (range 18-35), a mean body weight of 60.3 kg (range 50-68 kg), and a mean high of 169.3 cm (range 158-180 cm) were recruited as non-paid volunteers. All subjects were non-smokers except for one and in good health. They were not taking any medication at the time.

Each subject participated in four experimental sessions with at least one day apart. A small dose of alcohol (0.35-0.5 g/kg) was administered after the subjects had eaten a standardised breakfast. The ethanol (40% v/v) was diluted with orange juice to give a 20% v/v cocktail and consumed with 300 ml-commercial yogurt diluted with water (milk consistence) during 30 minutes period after breakfast. 15 minutes and 8 h later, a venous blood samples were drawn. In the next session, the subjects consumed the same volume and doses of ethanol, but with 300 ml of fermented yogurt instead of milk.

The meals were prepared from commonly available foodstuffs used for breakfast in Sweden and the individual components were chosen to contain low proportions of fat (20%) and protein (36%) and carbohydrate (46%), providing approximately 500 kcal of energy.

Figure 2B shows the individual BACs after treatment with fermented yogurt. In all three cases the yogurt pre-treatment had conferred substantial reductions in BAC levels, ranging from 58% to almost 80% in reduction, with a mean reduction of 69% in BAC.

Example 5. The effect of delayed administration of fermented yogurt on BAC in two volunteer persons

The effect of delayed ingestion of the fermented yogurt product on BAC was also investigated. At two different occasions, the two volunteer persons first ingested 250 ml alcohol (40% v/v) during a three-hour period, following dinner. By the end of the 3 hour extended period of alcohol consumption, the volunteer persons were given 500 ml of regular milk in a first session, respectively 500 ml of fermented yogurt product in a second (identical) session. The BAC values were measured after an additional 8 hours period. The results demonstrate that also delayed administration and ingestion of the fermented yogurt product affects the pharmacokinetics of ethanol absorption. Here, we

could show that the final BAC values was still reduced by more than 70% (mean reduction), Fig 3. The results suggest that the active components of the fermented yogurt product exhibit alcohol reducing and cleansing properties also when the alcohol has been absorbed from the GI-tract to the blood and systemic circulation.

Yet another mechanism which might explain the decreased bio-availability of ethanol when consumed together with fermented yogurt is the amplified action of gastric ADH. When ethanol remains in the stomach for a longer period of time, because of binding to constituents of the fermented yogurt, oxidation by gastric and bacterial ADH would be facilitated.

The results from Fig 3 suggest that alcohol can be metabolised and thus reduced from blood in the first passage metabolism (FPM), because of the induction of ADH and ALDH enzymes to the gastrointestinal epithelial lining by fermented yogurt. Therefore, the remained alcohol in stomach does not entrance into the systemic circulation. Alternatively, lactic acid might be absorbed to the systemic circulation and cause esterification of alcohol.

Example 6. The content of lactose and galactose in fermented yogurt product

The content of galactose and lactose in the fermented yogurt produced in Example 1 were analysed by use of ion-chromatography with electrochemical detection. The analysis was performed by AnalyCen Nordic AB, 404 29 Gothenburg, Sweden. The analyse method was done with high-performance liquid chromatography (A206:21, HPLC).

Lactase and galactase seem to be present in active forms since the level lactose and galactose were significantly decreased in fermented yogurt compared with casual yogurt. The concentrations of these carbohydrates were less than 0.4 g/L by using ion chromatography with electrochemical detection. The results suggested the corresponding enzymes lactase are present in active form and suggest the beneficially preparation of fermented yogurt product for lactase and galactase deficient individuals.

In individual suffering from galactosemia (a rare inherited inborn error of metabolism) free galactose may cause intolerance. We also analysed the content of lactose and galactose. In the fermented yogurt product the results showed 90% reduction of lactose and galactose which could be important factor for better tolerating fermented yogurt by lactase or galactase deficient individual.

Example 7. The content of lactic acid optical isomers (L + and D -) in fermented yogurt product

Lactic acid in fermented yogurt occurs in two optical isomers, L(+) and D(-). In order to prevent the appearance of metabolic acidosis due to the inability of the body to transform or metabolise sufficient amount of D(-) lactic acid, the content of L(+) and D(-) isomers were analysed by using spectophotometery analysis. The casual yogurt contain D(-) lactic acid (0.05 g/100g) and L(+) lactic acid (0.07 g/100g) compared to the fermented yogurt which contain higher amount of D (-) isomer, (0.47 g/100g).

Fermentation results in the conversion of some of the lactose into lactic acid. The World Health Organisation (WHO) recommends that no more than 100 mg D(-) lactic acid per kg body weight should be consumed daily (WHO, 1974). Our results suggest that one litre of liquid fermented yogurt daily is completely safe in a normal diet based on WHO recommendation 1974.

In the presence of D-lactate dehydrogenase (D-LDH), D-lactic acid (D-lactate) is oxidised by nicotinamide-adenine dinucleotide (NAD) to pyruvate. The oxidation of L-lactic acid requires the presence of the enzyme L-Lactate dehydrogenase (L-LDH).

$$\text{D-lactate} + NAD^+ \xrightarrow{\text{D-LDH}} \text{Pyruvate} + NADH + H^+$$

$$\text{L-lactate} + NAD^+ \xrightarrow{\text{L-LDH}} \text{Pyruvate} + NADH + H^+$$

The equilibrium of both these reactions are almost completely towards lactate. However, by trapping pyruvate in a subsequent reaction catalysed by the enzyme glutamate-pyruvate transaminase (GPT) in the presence of L-glutamate, the equilibrium can be displaced in favour of pyruvate and NADH. The amount of NADH formed in the above reactions is stoichiometric to the amount of D-lactic acid, respectively. The increase in NADH is determined by means of its light absorbance at 334, 340 or 360 nm. The kit and other compounds were obtained from Boehringer Mannheim, USA.

Example 8. Composition of fermented butter

Separation of butter from fermented yogurt.

The multi-cultured yogurt was fermented for one week. Butter was made by churning of liquid fermented yogurt until the fatty globules of butter separated from the suspension. The churning cream in form of butter was separated from the fermented suspension by shaking at 30°Cfor 24 hours. The butter constituency was analysed by gas-chromatography with capillary column and flame ionisation's detector (AnalyCen Nordic AB, 404 29 Gothenburg, Sweden).

Estimation of the Colony Forming Units (CFU), and homogeneous colony from original inoculum.

A serial dilution was made to estimate the number of living bacteria in the fermented yogurt and butter products. 1 ml samples were transferred to a tube containing 9 ml of sterile water and a serial dilution was made and inoculated on selective ROGOSA-plates for *Lactobacillus* and blood agar plates. The colony forming units was used to calculate the viability of the in-cultivated bacterial strains. The homogeneity of the colonies was confirmed by Gram staining.

Similar to the fermented yogurt the fermented butter contains live bacteria. By analysis of serial dilutions, 1.2×10^6 of colony forming units (cfu) per ml was found. This number of live bacteria corresponds to 5% of cfu compared to fermented yogurt (cfu; $2.4 * 10^7$). The composition of the fermented butter was also analysed by gas chromatography, where it

was shown that close to 30% of fatty acids were unsaturated while 66% were saturated fatty acids (Table 1).

Example 9. Effect of fermented yogurt or fermented butter on plasma lipid in five volunteers

The effect of long-term intake of fermented yogurt or fermented butter on serum lipid levels in five healthy, normolipidemic adult volunteers was investigated during the 4 weeks study. One person kept the casual and normal diet during the experiment and was used as a non-treated control. Two groups consisting of two volunteer each group supplemented their daily diet with 45 gram fermented butter or one litre fermented yogurt. The butter consists of 95% fat and live *Lactobacillus helveticus, Lactobacillus acidophilus* bacteria and yeast. Blood samples were drawn for analysis two times; before start of experimental diet and another sample taken after the 4 weeks period and analysed for total cholesterol, LDL, HDL and triglyceride. Fig 4 shows the serum-triglyceride levels of both groups (fermented yogurt and butter). Serum-triglyceride concentration was decreased with 18.4% (mean reduction) in the fermented yogurt group (IV and V), whereas the two persons in the fermented butter group showed an increase with 20% (mean increasing), after the 4 weeks period (II and III).

Interestingly, the Atherogenic Index (AI), [(total cholesterol – High-Density Lipoprotein cholesterol)/High-Density-Lipoprotein cholesterol] for both groups (butter and fermented yogurt groups) showed very small changes even after 4 weeks of consumption of butter (mean AI; from 2.97 to 3.02) or fermented yogurt (mean AI; from 2.13 to 2.05). The control showed an increase of triglyceride by 2% and almost no change in AI (AI; from 3.38 to 3.31), (Fig 5).

Here, it is shown that fermented yogurt can lower the blood concentration of triglycerides and replacement of fermented butter, as an alternative to common butter, does not increase the serum triglycerides level significantly. These results indicate the potential use of fermented butter or yogurt with both protective and therapeutic effects.

Independent of daily high intake of fermented butter or fermented yogurt, the atherogenic index were still constant (Fig 5).

Effect of fermented yogurt on serum-lipid concentration.

Two healthy females with a mean age 49.5 yr. (range 44-55), a mean body weight of 67.5 kg (range 60-75 kg), and a mean high of 161 cm (range 160-162 cm) were recruited as non-paid volunteers. All subjects were non-smokers and in good health. They were not taking any medication at the time.

One litre-fermented yogurt was administered diurnal for exactly 30 days. Before and after the end of experiment, a venous blood sample was drawn.

Two other healthy men and two females with a mean age 51.5 yr. (range 36-67), a mean body weight of 61.5 kg (range 54-69 kg), and a mean high of 157.5 cm (range 155-160 cm) were recruited as non-paid volunteers. All subjects were non-smokers and in good health. They were not taking any medication at the time.

45 gram (mean) fermented butter was consumed diurnal for exactly 30 days and the volunteer has kept the normal diet during the experiment. Before and the end of experiment, a venous blood sample was drawn.

Table 1.

Proximate composition, fatty acids of fermented butter prepared from fermented yogurt.

Common Name	Abbreviation	%-saturated	%-mono- di- tri- unsaturated
Caproic acid	6:0	1,8	
Caprylic acid	8:0	1,2	
Capric acid	10:0	2,9	
Lauric acid	12:0	3,7	
Myristic acid	14:0	11,1	
Myristoleinic acid	14:1		0,9
Pentadecaneic acid	15:0	0,9	
Palmitic acid	16:0	33,6	
Palmitoleic acid	16:1		1,5
Margarine acid	17:0	0,4	
Heptadecanic acid	17:1		0,2
Stearic acid	18:0	10,1	
Oleic acid	18:1		22,7

Linoleic acid	18:2		2,5
Alfa-linolenic	18:3		0,4
Octadecatetraic acid	18:4		0,1
Arachidic acid	20:0	0,2	
Gadoleic acid	20:1		0,2
Elcosadienic acid	20:3,4		0,2
EPA		20:5	0,1
Behenic acid	22:5	0,1	
Total		65,9%	28,9%

The purity of the analysed butter was 94,8%.

References

1- Blanc, B., (1973). *Schweiz. Mildchztg.***99:**463.

2. Department of Food Engineering, Trakya University, Agriculture Faculty of Tekirdag, Turkey, Tarhana as a traditional Turkish fermented cereal food, its recipe, production and composition, Nahrung 2000, Apr; 44(2): 84-8.

3- Jones, A.W. (1991) Limits of detection and quantification of ethanol in specimens of whole blood from drinking drivers analyzed by head space gas chromatography. *J Forensic Sci***36:** 1277-1279.

4- Mann, G.V. and Spoerry, A. (1972). Atherosclerosis in the Masai.*Amer J Clin Nutr* **27:**464.

5- Mann, G.V . (1977). A factor in yogurt which lowers cholesteremia in man. *Artherosclerosis*, **26:**335.

6- Niemela, O., Parkkila, S., Pasanen, M., Iimuro, Y., Bradford, B., Thurman, R.G. (1998), Early alcoholic liver injury: formation of protein adducts with acetaldehyde and lipid peroxidation products, and expression of CYP2E1 and CYP3A. *Alcohol Clin Exp Res***22(9):** 2118-2124.

7- Ponnappa, B. C., Rubin, E. (2000) Modeling alcohol effect's on organs in animal models. *Alcohol research & Health***24(2):**93-103.

8- Rasic, J. and Kurman, J.A. (1978). Yogurt-Scientific Grounds, Technology, Manufacture and Preparation. *Dairy Publishing House, Copenhagen, Denmark.*

9- Widmark, E.M.P. (1936) Undersökningar över födans inflytande på etylalkoholens omsättning. *Kungl Fysiografiska Sällskapet i Lund Förhandlingar***9:** 279-307.

10- Widmark, E.M.P. (1933) Der Einfluss der Nahrungsbestandteile auf der Alkoholgehalt des Blutes. *Biochem Z***267:**135-142.

CLAIMS

1. Fermented milk product, comprising bacteria from the Lactobacillus species and yeast having a galactose content of less than 1,2 g/l, a lactose content of less than 1,2 g/l, and lactic acid content of at least 0.2 weight %.

2. Fermented milk product according to claim 1, characterised in that the galactose content is less than 0,4 g/l and the lactose content of less than 0,4 g/l.

3. Fermented milk product according to claim 1 and/or 2, characterised in that the Lactobacillus species is chosen from *L. helveticus*(AD4550/01 DSM 14492)and *L. acidophilus*(Lb. 67 DSM 14499).

4. Fermented milk product according to any of claims 1 -3, characterised in that the product is yogurt, butter or cheese.

5. A method for producing a fermented milk product, comprising bacteria from the Lactobacillus species and yeast having a unique combination of galactose content of less than 1,2 g/l, a lactose content of less than 1,2 g/l, and lactic acid content of at least 0.2 weight %, characterised in that milk is heated, Lactobacillus bacteria are added and the bacterial suspension is incubated at 25-30, preferably at 37°C for 2-24, preferably 12 hours in normal atmosphere, the yogurt produced diluted with water to drive the fermentation processes further and additional Lactobacillus bacteria and *yeast* is added and the suspension is incubated at 15-25 °C, preferably at room temperature for 1- 15, days, preferably 7 days in a closed system.

6. Use of a fermented milk product according to any of claims 1- 5, for the production of a pharmaceutical for lowering of plasma triglycerides and lipids.

7. Use of a fermented milk product according to any of claims 1- 5, for the production of a pharmaceutical or probiotic product for hampering and/or profylax of cell death, especially caused by the consumption of alcohol.

8. Use of a fermented milk product according to any of claims 1- 5, for the production of a pharmaceutical or probiotic product for hampering and/or profylax of fatty-liver, especially caused by the consumption of alcohol.

9. Use of a fermented milk product according to any of claims 1- 5, for the production of a pharmaceutical or probiotic product for profylax and treatment of atherosclerosis and coronary heart disease.

10. Use of a fermented milk product according to any of claims 1- 5, for the production of a pharmaceutical or probiotic product for profylax and treatment of lactase and galactase deficient individuals.

11. A symbiotic or functional food product comprising a fermented milk product according to any of claims 1- 5 reducing alcohol content in the GI-tract, in the blood and in the systemic circulation in humans.

Figure 1A

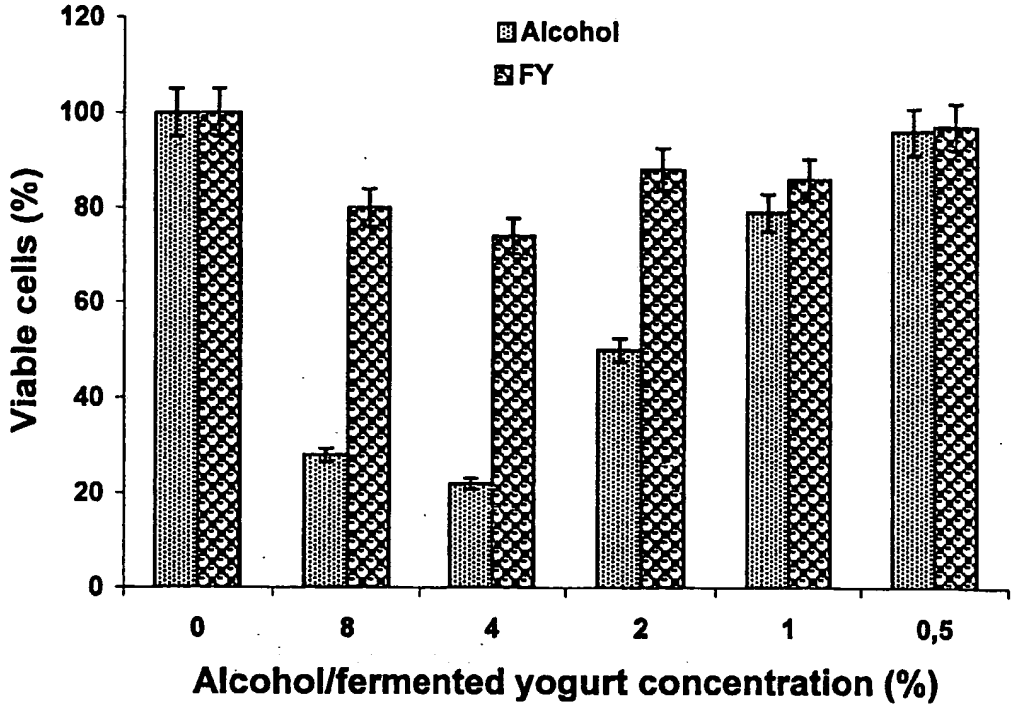


Figure 1B

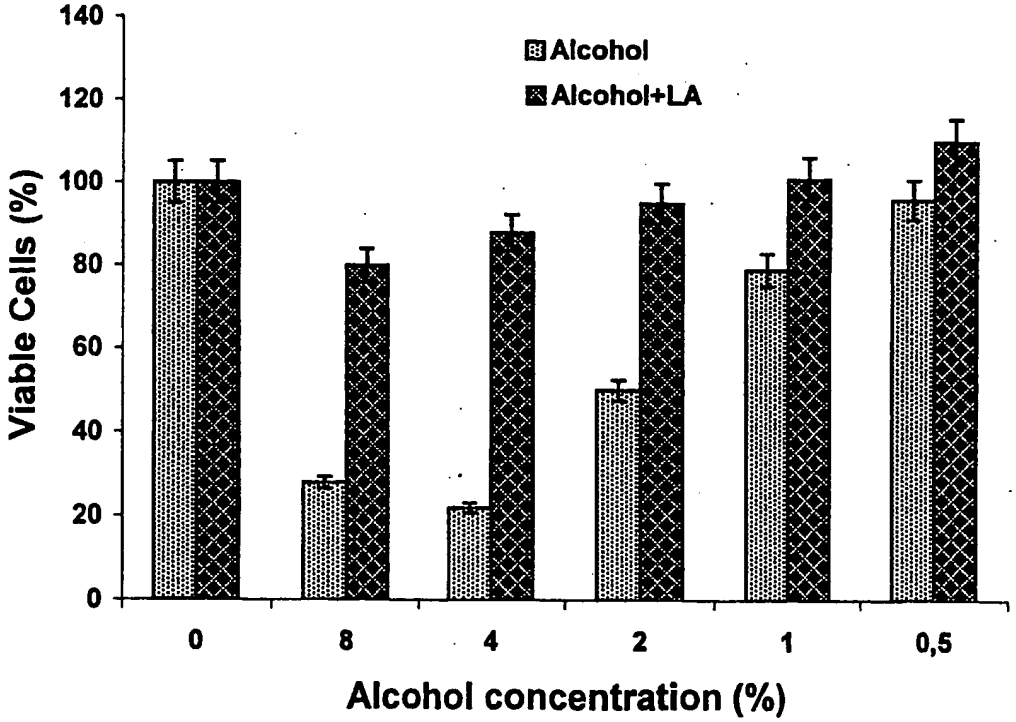


Figure 1C

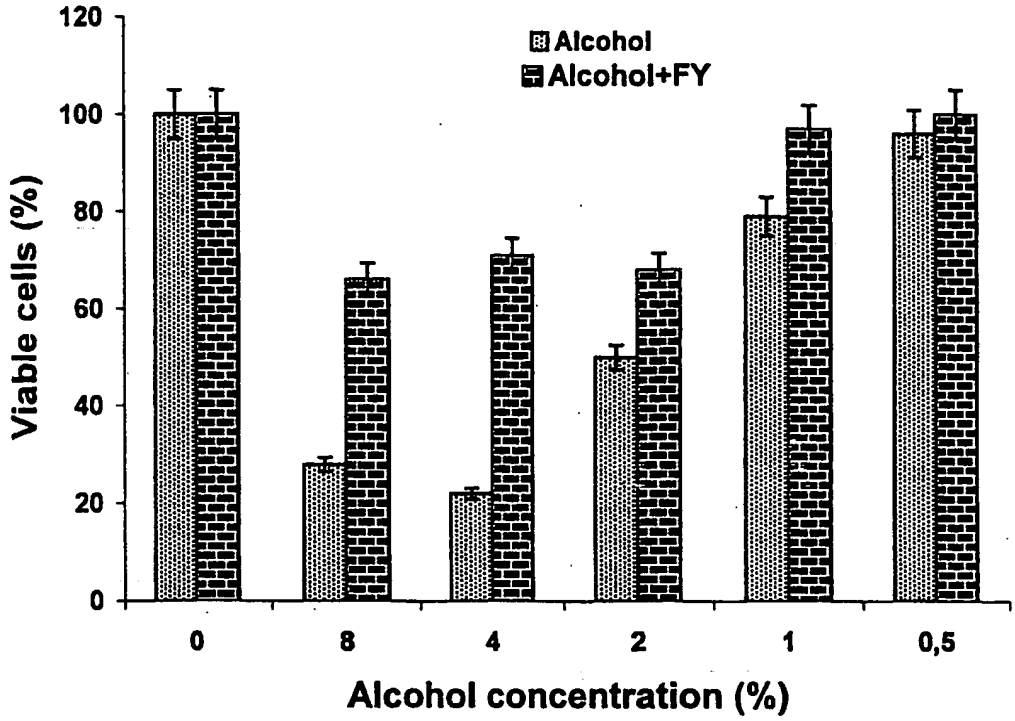


Figure 2A

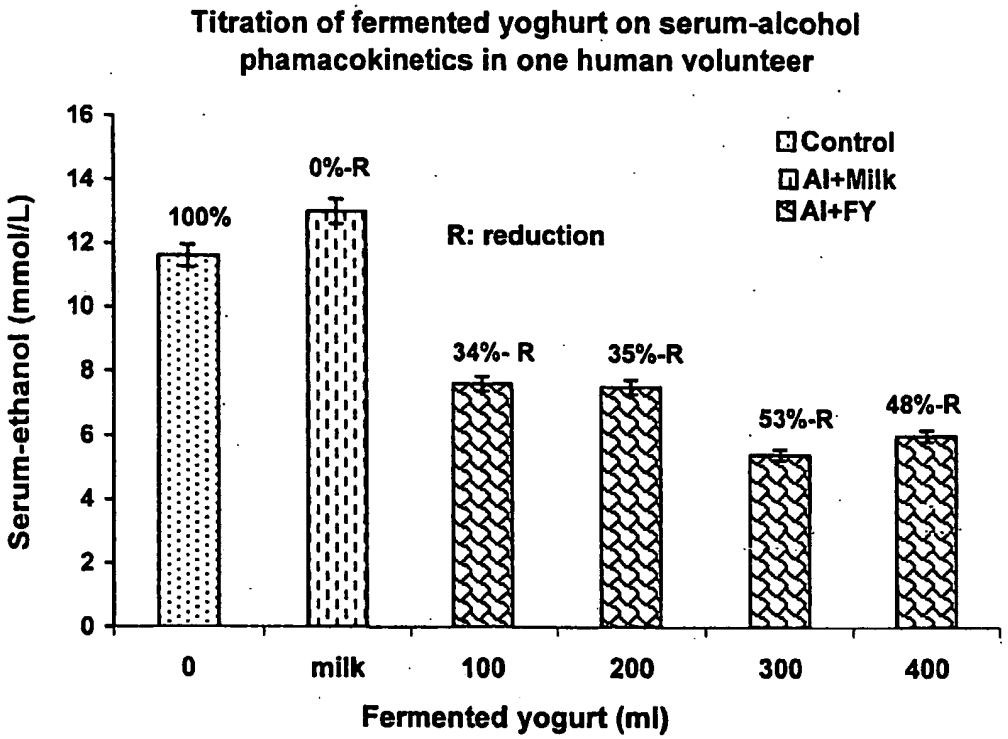


Figure 2B

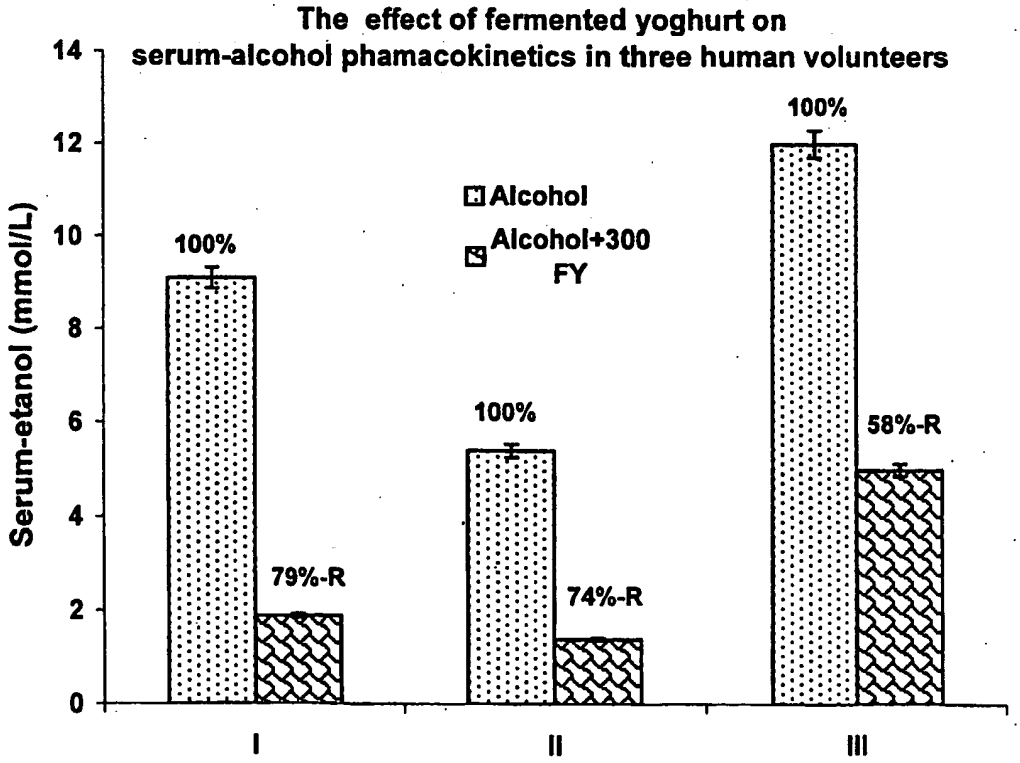


Figure 3

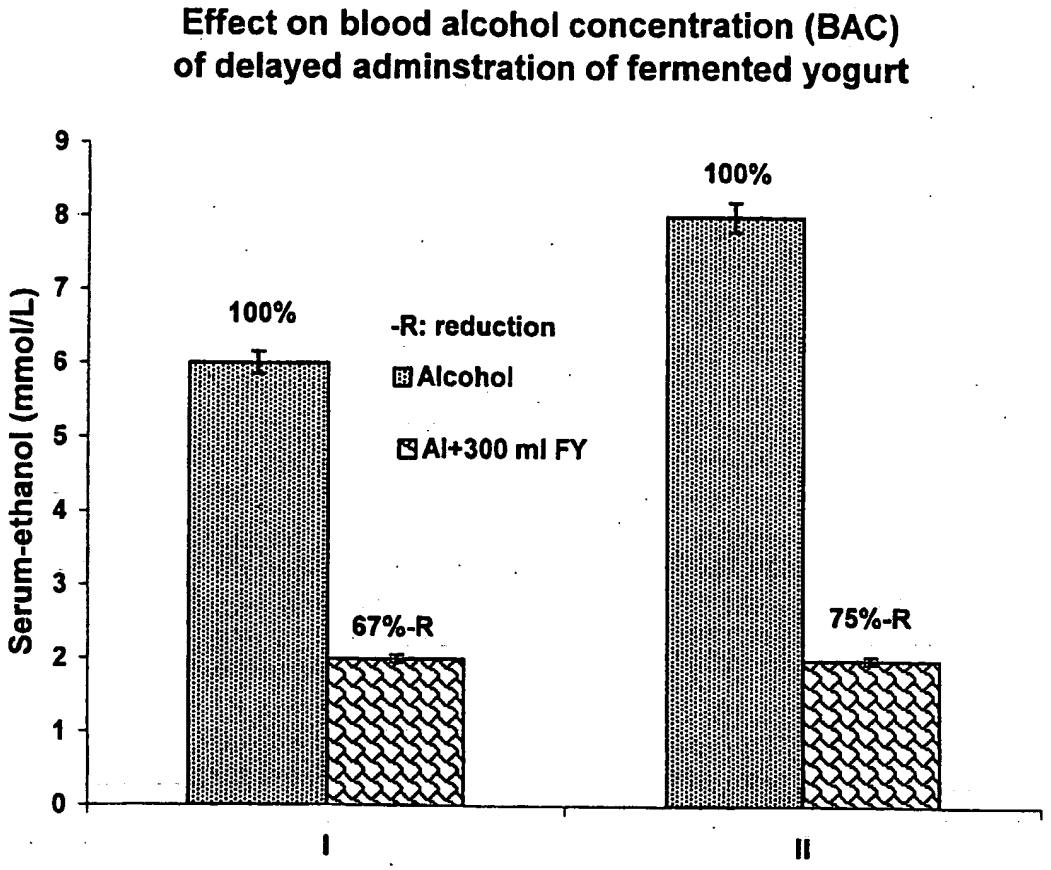


Figure 4

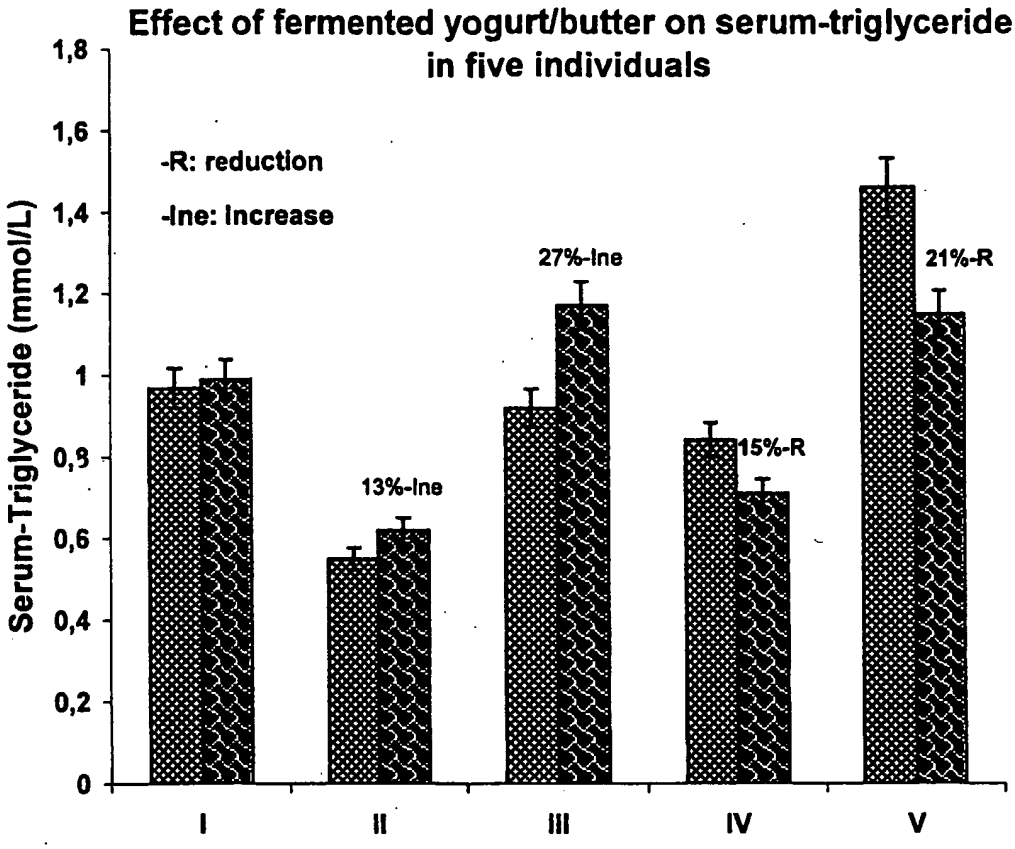
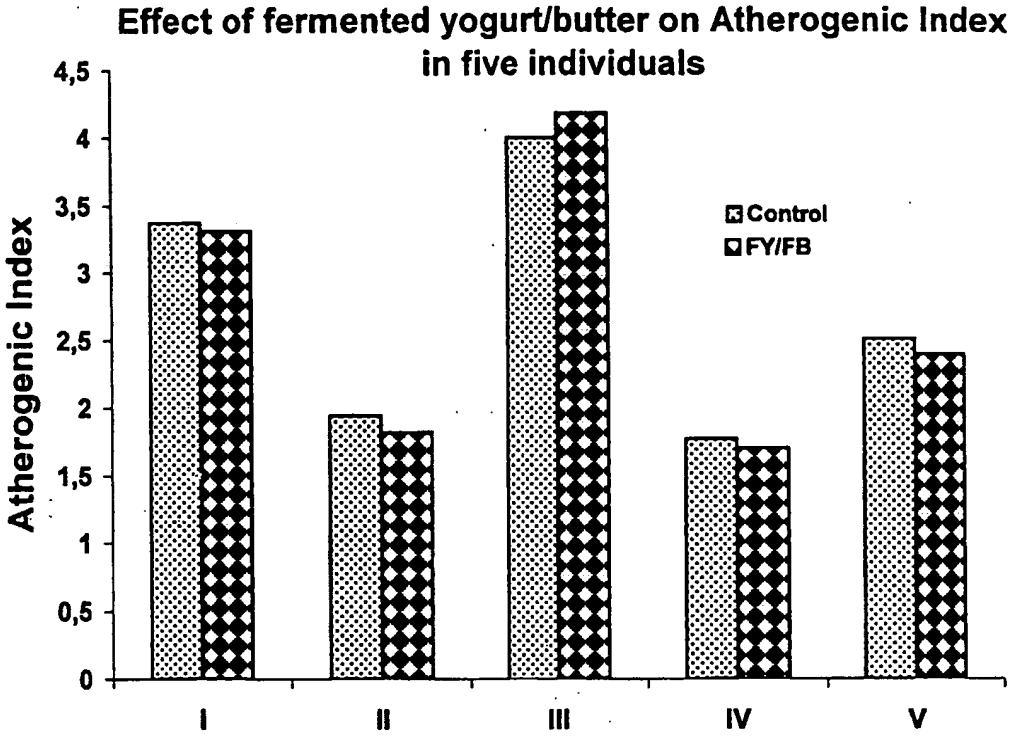


Figure 5



INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 03/00632

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: A23C 9/127, A23C 19/032 // (C12N 1/20, C12R 1:225, C12R 1:23)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: A23C, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, MEDLINE, BIOSIS, CA, FROSTI, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 0060950 A1 (SIGMA-TAU HEALTHSCIENCE S.P.A.), 19 October 2000 (19.10.00), page 6 - page 7	1-6,9
A	STN International, file CAPLUS, CAPLUS accession no. 1980:469086, Document no. 93:69086, Kashiyama, Shinkei: "Candida krusei and fermented product produced by culturing the yeast and lactic acid bacterium", JP,A2,55026827, 19800226	1-4
A	EP 0122104 A2 (ROBERTS, JAMES GORDON), 17 October 1984 (17.10.84)	1-11

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 August 2003	15 -08- 2003
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Yvonne Siösteen/Els Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 03/00632

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	National Library of Medicine (NLM), file Medline, Medline accession no. 10067658, Anderson J W et al: "Effect of fermented milk (yogurt) containing Lactobacillus acidophilus L1 on serum cholesterol in hypercholesterolemic humans"& Journal of the American College of Nutrition, volume 18, no. 1,Februari 1999, pages 43 - 50 -- --------	6,9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 03/00632

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0060950 A1	19/10/00	AU 4311800 A	14/11/00
		BR 0006040 A	13/03/01
		CA 2334877 A	19/10/00
		CN 1300187 T	20/06/01
		EE 200000713 A	15/04/02
		EP 1085816 A	28/03/01
		HU 0103895 A	28/02/02
		IL 139853 D	00/00/00
		JP 2002540807 T	03/12/02
		NO 20006284 A	29/01/01
		PL 344670 A	19/11/01
		SK 18862000 A	11/06/01
		TR 200003689 T	00/00/00
		US 6511685 B	28/01/03
EP 0122104 A2	17/10/84	CA 1248816 A	17/01/89
		DE 3404474 A	11/10/84
		IL 71423 A	31/01/86
		JP 60041441 A	05/03/85
		NO 841333 A	08/10/84